Biochemical Studies on the Membranes as A Mechanism of Toxicity of Hexachlorocyclohexane

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BY

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LUCKNOW - 226001

1995

Dedicated

To

MY RESPECTED PARENTS

With

LOVE & AFFECTION

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CERTIFICATE

This is to certify that the work embodied in this thesis entitled "**BIOCHEMICAL STUDIES ON THE MEMBRANES AS A MECHANISM OF** TOXICITY OF HEXACHLOROCYCLOHEXANE" has been carried out by **Mr.Farrukh Afaq** under my supervision.

He has fulfilled the requirments of the Aligarh Muslim University for the degree of **Master of Philosophy in Biochemistry.**

The work included in this thesis is original unless stated otherwise and has not been submitted for any other degree.

Deepa Agrawal
(DEEPA AGRAWAL)

A C K N O W L E D G E M E N T S

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C O N T E N T S

Preface

Hexachlorocyclohexane (HCH) is a stable and persistant organochlorine insecticide extensively used in agriculture. Human exposure to hexachlorocyclohexane occurs primarily by occupational exposure, by ingesting HCH in contaminated food or water. HCH is known to cause peripheral neuropathies, abnormal electroencephalographic changes, convulsions, seizures, toxic hepatitis and blood anomalies.

The erythrocyte is a convenient model to study xenobiotic induced membrane damage leading to hemolysis and leaking out of molecular constituents. The erythrocyte membrane provides important example of membrane organization and cytoskeleton attachment, and is frequently implicated to have a primary role in the intoxication and storage process of insecticides. The permeability properties of biomembranes are regulated by the physical and functional state of membrane components which modulate lipid-lipid, lipid-protein and protein-protein interactions. In recent years, considerable amount of new

information has been accumulated regarding the molecular architecture and functional mechanistics of erythrocyte membrane, such as specific membrane proteins, transmembrane signals, pumps and chemicals. This raises the need for detailed studies of mechanisms of membrane damage by toxicants and which could possibly be used for clinical diagnostic purposes in human health.

ABBREVIATIONS

AchE	:	Acetylcholinesterase
ATPase	:	Adenosine triphosphatase
ATP	:	Adenosine triphosphate
$CaCl_2$	:	Calcium Chloride
Conc.	:	Concentration
^oC	:	Degree centrigrade
DTNB	:	5,5'-dithiobis-2-nitrobenzoic acid
EDTA	:	Ethylenediamine tetraacetic acid
EGTA	:	Ethylene glycol bis tetraacetic acid
Fig.	:	Figure
GSH	:	Reduced glutathione
g	:	Acceleration due to gravity
HCH	:	Hexachlorocyclohexane
HCl	:	Hydrochloric acid
h	:	hour
KCl	:	Potassium chloride
LD	:	Lethal dose
$MgCl_2$	:	Magnesium chloride
MDA	:	Malondialdehyde
min	:	minute
O.D.	:	Optical density
PA	:	Phosphatidic acid
PI	:	Phosphatidyl inositol
PIP	:	Phosphatidylinositol 4-phosphate
PIP_2	:	Phosphatidylinositol 4,5-bisphosphate
Pi	:	Inorganic phosphate
RBC	:	Red blood cell

SDS	:	Sodium dodecyl sulphate
S.E.	:	Standard error
TBA	:	2-Thiobarbituric acid
TBARS	:	Thiobarbituric acid reactive substances
TCA	:	Trichloroacetic acid
Temed	:	Tetramethyl ethylene diamine
TLC	:	Thin layer chromatography
v/v	:	Volume by volume
w/v	:	Weight by volume

Introduction

Pesticides are biologically active compounds intended mainly to kill pest population by their toxic and deliterious actions. Pesticides are defined under the Federal Insecticide, Fungicide and Rodenticide Act as amended; they include "any substance or mixture of substances intended for preventing, destroying, repelling or mitigating any pest [insect, rodent, nematode, fungus, weed, other forms of terrestrial or aquatic plant or animal life or viruses, bacteria, or other micro-organisms, except viruses, bacteria, or other micro-organisms on or in living man or other animals, which the Administrator declares to be a pest] and any substance or mixture of substances intended for use as a plant regulator, defoliant or desiccant" (Hayes, 1982). The important contribution of pesticides to our health and economy prompt their continued use for decades. In developing countries like India, chemical control of pests has led to dramatic increase in food production and supression of human diseases, such as malaria, filariasis

and viral encephalitis etc. Besides controlling pest, pesticides have become hazardous to both lives and environment. Pesticides have become the most alarming among environmental pollutants posing a great risk to agriculture workers and general public. Therefore complete knowledge of their toxicity is essential to ensure their safer use and to reduce their environmental and human health hazards. The present study was undertaken by using hexachlorocyclohexane (HCH) an organochlorine insecticides.

Hexachlorocyclohexane (HCH) $C_6H_6Cl_6$

Hexachlorocyclohexane was discovered in 1825, but its insecticidal properties were first patented only in the 1940s. It has been produced commercially since 1949 and has molecular weight 290.8. The structures of HCH is shown (Fig. 1). Hexachlorocyclohexane is a synthetic chemical that exists in many chemical forms (called isomers). These isomers are alpha, beta, gamma, delta, epsilon and zeta.

Technical grade HCH is synthesized from benzene and chlorine in the presence of ultraviolet light and comprises 65-70% alpha HCH, 7-10% beta HCH, 14-15% gamma HCH (lindane) approximately 7-10% delta HCH, 1-2% epsilon HCH, and 1-2% other components. With respect to acute exposure, gamma-HCH is the most toxic, followed by alpha-, delta-, and beta HCH. However on chronic exposure beta - HCH is the most toxic followed by alpha, gamma and delta HCH. The increased toxicity of beta-HCH is probably due to its longer half life in the body and its accumulation in the body with time.

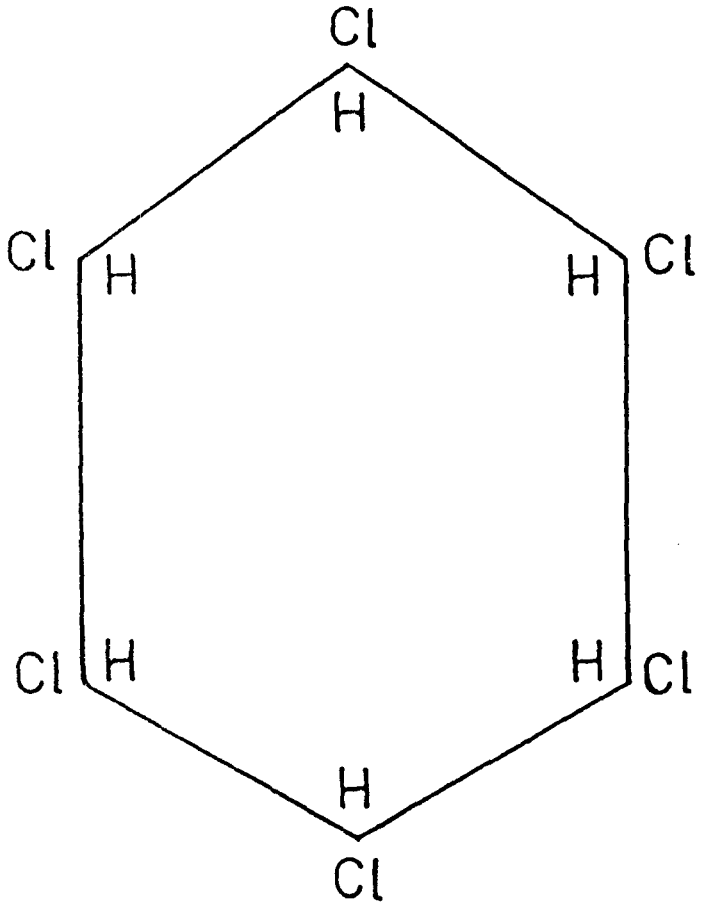


Fig. 1: STRUCTURE OF HCH

Hexachlorocyclohexane is an organochlorine which is mainly used as contact fumigant pesticide. HCH can be absorbed by human skin and is an irritant to both skin and eyes. Human exposure to HCH occurs primarily by occupational exposure, ingesting HCH contaminated food or water or through the misuse of therapeutic lotions containing gamma-HCH used to control mites or lice. Occupational exposure causes nervous system dysfunctions, metabolic disorders and vasomotor paroxyms. The depression can lead to respiratory failure. HCH when administered to rat and mouse produces liver tumors. In general HCH is highly toxic by ingestion and moderately toxic by inhalation and dermal exposure. HCH is stable in air, unaffected by light, heat and CO_2. The mixture is moderately soluble in acetone, benzene and chlorinated hydrocarbon solvents. It is poorly soluble in kerosene only slightly soluble in fats and oils, and almost insoluble in H_2O. Its persistence in the environment and accumulation in mammalian tissue has caused great concern to humans.

The detoxification of HCH appears to be dependent on P-450 oxidative system. The proposed metabolism of hexachlorocyclohexane is shown (Fig. 2).

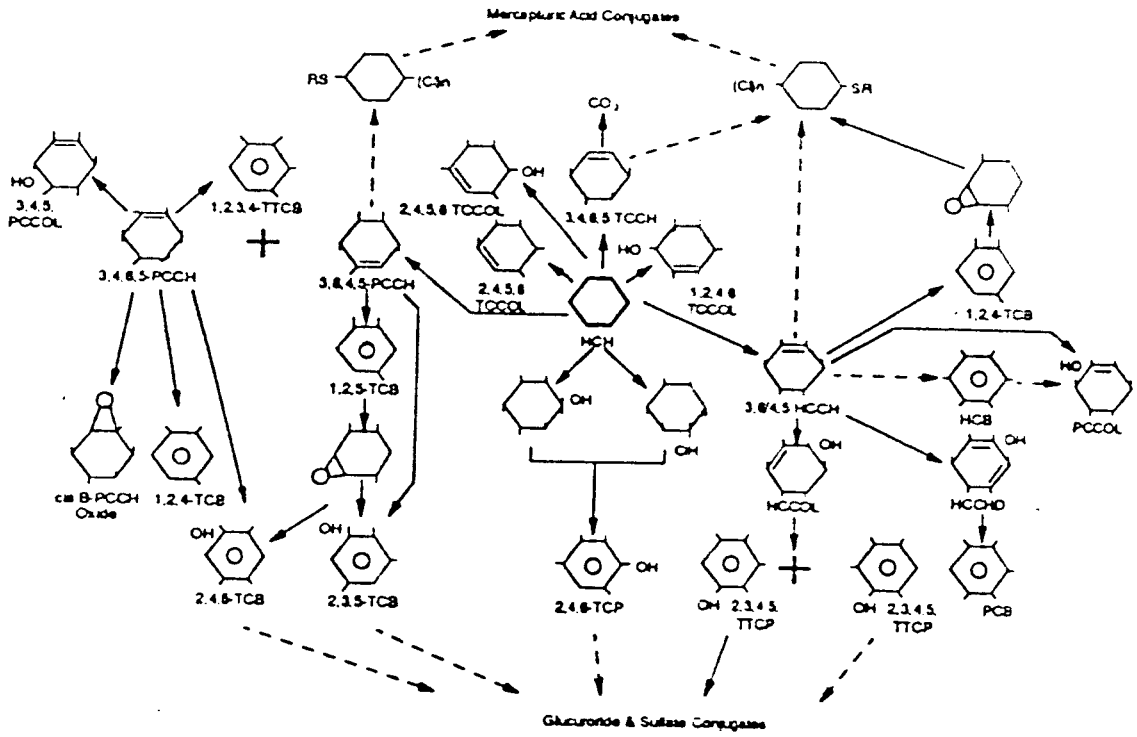
The erythrocyte membrane

The best characterized cell membrane is the one surrounding the mammalian red blood cell, or erythrocyte. The erythrocyte has no nucleus and contains no intracellular organelles. Especially a bag of hemoglobin with relatively few other proteins, its function is to transport oxygen from the lungs to the tissues and carbon dioxide back to the lungs.

FIGURE : 2. The Proposed Metabolism of Hexachlorocyclohexane

Abbreviations:

HCCH:	Hexacholorocyclohexene
HCB:	Hexachlorobenzene
HCCHD:	Hexachlorocyclohecadiene
HCCOL:	Hexachlorocyclohexunol
HCH:	Hexachlorocyclohexane
PCCOL:	Pentachlorocyclohexenol
PCCH:	Pentachlorocyclohexene
PCB:	Pentachlorobenzene
TCCOL:	Tetrachlorocyclohexenal
TCCH:	Tetrachlorocyclohexene
TTCB:	Tetrachlorobenzene
TTCP:	Tetrachlorophenol
TCB:	Trichlorobenzene
TCP:	Trichlorophenol
3,6/4,5-HCCH:	3,6/4,5-Hexachlorocyclohexene



* Source: Chadwick et al. 1979, 1985; Fitzloff and Pan 1984; Fitzloff et al. 1982.

The erythrocyte membrane contains proteins as well as lipids. A current view of how proteins are distributed in the plasma membrane of red blood cell is shown (Fig. 3). The erythrocyte membrane is homogenous. Its proteins appear to be more or less uniformly distributed in the plane of the membrane, without large specialized patches. The red cell is also unusual among mammalian cells because its cytoskeleton forms a cell that lies under the entire plasma membrane and is attached to it at many points. This structure gives the membrane great strength and flexibility. The erythrocyte membrane provides important examples of membrane organization and cytoskeleton attachement. The erythrocyte membrane is 52% protein, 40% lipid and 8% carbohydrate. 93% carbohydrate (as oligosaccharides) is attached to protein, forming glycoprotein and 7 percent to lipids, forming glycolipids.

The erythrocyte have two main integral proteins, glycophorin and band 3 (also known as anion channel). These are glycoproteins. The predominent extrinsic protein of erythrocyte membrane are band 1 and 2 (spectrin), band 2.1 (ankyrin), band 4.1 and band 5 (actin). These five proteins are held together by non-covalent bonds and they form the strong but flexible frame work responsible for the shape and pliability of the red blood cell.

The major constituents of the cytoskeleton are the α and β spectrin (band 1 and 2). The two spectrin chains combine to form dimers that are long (100 nm), slender (5 nm diameter), wormlike structure in which the subunits coil about each other. Two spectrin $\alpha\beta$ dimers combine head to head to form an $(\alpha\beta)_2$

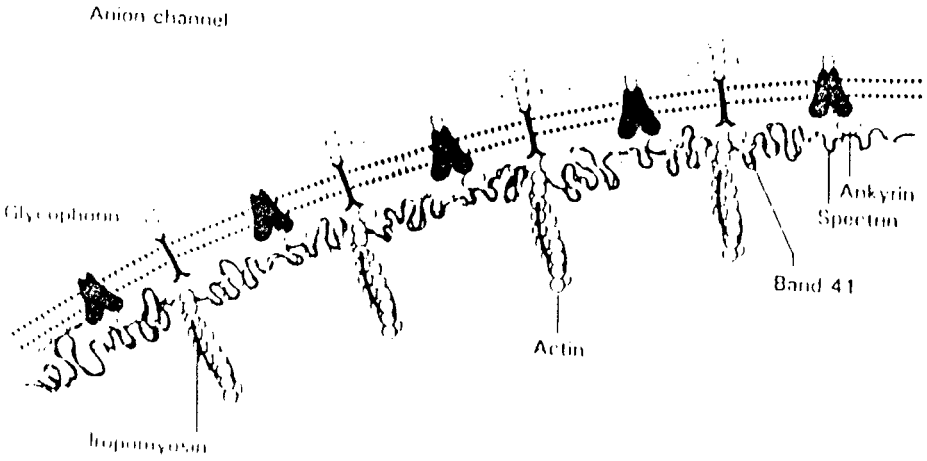


Fig.:3. **Organization of the erythrocyte cytoskeletal filaments.** (Source: Fowler,V.M. 1986)

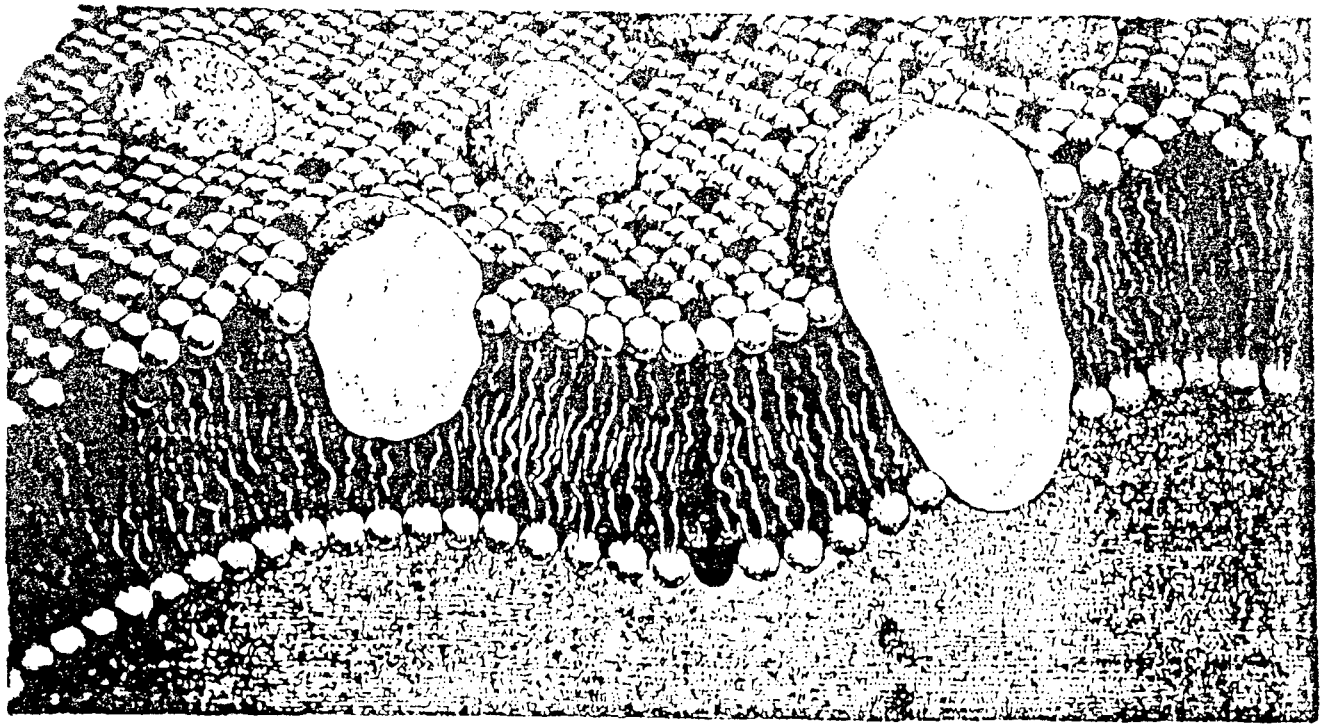


Fig:4. Three dimensional fluid mosaic concept of membrane structure. The solid bodies represent the globular integral proteins which at long range are randomly distributed in the plane of the membrane. The open circles denote the ionic and polar head groups of the phospholipid molecules which make contact with water and wavy lines.

tetramer, this interaction can take place in an aqueous solution. Each erythrocyte ghost contains about 200,000 spectrin dimers. They form the lace like fibres of the cytoskeleton and are bound to the cytoplasmic face of the erythrocyte membrane by two different types of protein-protein interactions.

In the cytoskeleton network, the free end of several spectrin tetramer are held together by the fibrillar chains of the protein actin, band 5. Actin plays a crucial role in cell movement. Band 4.1 lies at the actin-spectrin junction and helps join the actin to the spectrin. Because spectrin binds to the side of actin filaments, a single fibre of actin has many potential binding sites for spectrin. It offers junction points for three or more spectrin molecules and thus enables a network to form.

The interactions described thus far produce a lace like cytoskeleton but do not attach to the erythrocyte membrane. This attachement is mediated through the protein called ankyrin (band 2.1). The structure of erythrocyte membrane is shown (Fig. 4).

Review of Literature

Hexachlorocyclohexane (HCH) is a stable and persistent organochlorine insecticide extensively used in agriculture (Hayes, 1982). HCH is widely used in India and represents 50% of the total pesticides manufactured, the annual production being 40 million kilogram (Nigam *et al.*, 1986). HCH are chlorinated analogs of inositol; the alpha, beta, gamma and delta isomers of HCH have the stereochemical configurations of (+)-. scyllo-, muco-, and myo-inositol respectively. Human exposure is possible since it is used in pharmacological preparations and in public health for forest control (Reuber, 1979). HCH is known to cause neurological disorders such as peripheral neuropathies and abnormal electroencephalographic changes in workers (Burchfiel *et al.*, 1979; Anand *et al.*, 1980). Workers occupationally exposed to chlorinated pesticides develop toxic hepatitis and blood anomalies (Sasinovich *et al.*, 1974). The acute toxicity of lindane produces CNS excitation, convulsions and seizures (McNamara and Krop, 1948; Hulth *et al.*,

1978; Stark *et al.*, 1983). Liver necrosis and steatosis have been described among the hazards following acute and chronic lindane intoxication. HCH has low acute toxicity but high chronic toxicity mainly due to accumulation and slow degradation of its β-isomer (Baumann *et al.*, 1980).

Pesticides lower the capacity of erythrocytes to transport oxygen (Sikka *et al.*, 1974), alter cell membrane permeability (Kumar *et al.*, 1975; Sikka *et al.*, 1976) and cause changes in cell shape (Nishihare and Utsumi 1983; Gendel *et al.*, 1984; Denul *et al.*, 1986) thus making them more fragile and susceptible to hemolysis (Kobayashi *et al.*, 1979; Misra *et al.*, 1982; Agrawal *et al.*, 1989). Human erythrocytes depleted of ATP lose their smooth discoidal shape and adopt a spiny crenated form which coincide with the conversion of phosphatidyl-inositol 4,5-bisphosphate to inositol triphosphate and diacylglycerol. Phosphatidyl-inositol metabolism has been implicated in maintaining the shape of erythrocyte. The metabolism of inositol phospholipid is stimulated in many cells in response to a wide variety of hormones, neurotransmitters and other agents (Michell 1975; Irvine *et al.*, 1982). Y-and δ-HCH have been used in a number of intact cell studies to inhibit the synthesis of phosphatidyl inositol (Fisher and Mueller, 1971; Hoffmann *et al.*, 1980; Vu *et al.*, 1983). Y-HCH and δ-HCH which have the same configuration as muco-and myo-inositol (hexachlorocyclo-hexane) have been reported to inhibit the acetylcholine stimulated synthesis of phosphatidylinositol in guinea pig cerebral cortex slices and in rat embryo fibroblasts respectively. The insecticide lindane (γ-isomer of HCH) has

been reported to inhibit phytohemagglutinin stimulated phosphatidylinositol turnover (Fischer & Mueller, 1971) and to block acetylcholine stimulated phosphatidyl synthesis in cerebral cortex slices (Hokin & Brown, 1969). Phosphoinositide lipids are implicated in the receptor controlled mediation of cell responses to various stimuli although this class of lipids represent only a minor part of the total cell lipids and they have been extensively studied because of their specific biochemical properties (Hokin, 1985). The phosphoinositides derived second messengers transduce the signals of many hormones, growth factors and neurotransmitters into appropriate cellular responses (Hokin, 1985; Berridge, 1987; Subramoniam, 1988). Furthermore phosphoinositides are involved in membrane transport and microfilament dynamics (Michell, 1975; Subramoniam, 1988; Fisher *et al.*, 1992; Fukami, 1992). Many cell surface proteins are anchored to the membrane through phosphatidylinositol (Fisher *et al.*, 1992). The polyphosphoinositides located in the inner leaflet of plasma membrane have a very fast turnover rate (Michell, 1975; Hokin, 1985) and their levels in the cells could be an index of cell as well as membrane function. Previous studies showed that the structure and certain functions of erythrocyte membrane undergo significant changes as a consequence of HCH exposure (Agrawal *et al.*, 1989; 1991) raising the possibility of the involvement of phosphoinositide system. Elevated levels of diacylglycerol were found in echinocytes formed by calcium loading which activates a phospholipase C resulting in the degradation of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-

bisphosphate (Allan and Michell 1975) leading to change in K^+ efflux (Allan and Thomas, 1981).

Ca^{2+}-ATPase, the membrane bound enzyme responsible for transport of calcium out of the cell is membrane phospholipid dependent enzyme and has been shown to be markedly stimulated in the presence of phosphatidyl inositol 4-phosphate and phosphatidyl inositol 4,5-bisphosphate (Choquette *et al.*, 1984). The functional importance of Ca^{2+}-ATPase in maintaining the Ca^{2+}-levels in cell below 10^{-6} has been established (Schatzmann, 1973). Effect of lindane and lindane metabolites on microsomal and mitochondrial ATPases was observed (Heinvetter *et al.*, 1985). Increased hemolysis as a result of cellular injury has been associated with lipid peroxidation of red cells after paraquat treatment (Gabrylak and Klekot 1985; Heinvetter *et al.*, 1985). Free radicals can be produced by the reductive activity of cytochrome P-450 during Y-HCH bio-transformation (Baker *et al.*, 1985), as a result lipid peroxidation can occur. A dose dependent increase in lipid peroxidation of liver homogenate was observed after lindane treatment *in vivo* (Junqueira *et al.*, 1986). In contrast HCH (technical) showed an inhibition in lipid peroxidation of rat RBC under *in vitro* condition (Agrawal *et al.*, 1989). Cyclohexane radicals were detected after HCH exposure to isolated hepatocytes (Yvelin *et al.*, 1984). Formation of chlorobenzene and benzene by reductive dehalogenation of lindane in rat liver microsomes and the possibility of pentacyclohexane radical formations have been described (Baker *et al.*, 1985). Lipid peroxidation which requires the participation of free radicals has been proposed as

a major molecular mechanism involved in tissue injury, cancer, heart disease and aging. It is known that a large number of foreign compounds including environmental pollutants (Bus *et al.*, 1976; Albano *et al.*, 1982; McCay *et al.*, 1984) express their toxic effects through free radical generation mostly as active oxygen species. The mechanism of action of free radicals in pathogenesis of human diseases (Chance *et al.*, 1979; Davies *et al.*, 1982) have focussed on their ability to induce lipid peroxidation in cell or organelle membrane (Kollogg and Fridovich, 1977). It has been shown that lipid radicals and lipid hydroperoxides generated by xenobiotic compounds are responsible for degradation of proteins. Lipid peroxidation is toxic to cell as it initiates chain reactions, direct toxicity of lipid breakdown products, alterations of membrane fluidity, effect the metabolism of toxic compounds and also influence proteins function and protein susceptibility (Weitzman and Weitberg, 1985; Wolff *et al.*, 1986).

Glutathione (GSH) is an endogenous substrate able to react with a variety of electrophilic compounds protecting vital organs against toxic actions (Simplico *et al.*, 1984). Carter (1973) has reported that free sulphydryl groups play an important role in maintaining the structural integrity of the red cells. Levels of GSH are regarded as an index of vulnerability since binding of toxic chemicals to cellular structures and eventual cell death follow its depletion in parenchymal organs (Gillette *et al.*, 1974; Kuo and Hook, 1982).

Aims and Objectives

The aim of the present study is to explore the possibility of using RBC and other membrane systems in order to get clearer insight of membrane toxicity to pesticides. The effect will be observed *in vivo*, after hexachlorocyclohexane exposure.

Phosphoinositide metabolism is one such area which has been implicated in maintaining the shape of the membrane and for proper functioning of the carrier receptors on the membrane. Phosphoinositide lipids represent only a minor part of the total cell lipids and have been extensively studied because of their specific biochemical properties. Polyphosphoinositides (PIP+PIP_2) located in the inner leaflet of the plasma membrane have a very fast turnover rate and their levels in the cells could be an index of cell as well as membrane function. Previous studies have shown that the structure and certain function of erythrocyte membranes undergo significant change as a consequence of HCH exposure raising the possibilities of the involvement of phosphoinositide system. Therefore, studies were

undertaken to examine the steady state level and/or rate of formation of phosphoinositide in the erythrocyte membranes of hexachlorocyclohexane challanged rate. Studies were also conducted in brain order to correlate observed data with neurological symptoms.

Ca^{2+}-ATPase the membrane bound enzyme responsible for transport of calcium has been shown to be markedly stimulated in the presence of phosphatidylinositol 4,5-bisphosphate. Therefore studies were done on Ca^{2+}-ATPase also.

The other parameters studied were acetylcholinesterase in erythrocyte membrane, glutathione in blood, lipid peroxidation in liver, osmotic fragility in RBC, and erythrocyte membrane proteins were separated by SDS-PAGE.

Materials and Methods

Materials :

Bovine serum albumin, adenosine triphosphate were procured from Sigma Chemical Company, St. Louis, U.S.A. [2-^{3}H]-Inositol was purchased from Amersham, U.K., 5,5'-dithiobis-2-nitrobenzoic acid was purchased from Merck. 2-thiobarbituric acid was obtained from BDH Chemical Limited, Poole, England. All other reagents and chemicals used were of pure analytical grade, locally available.

Animals :

Male albino rats (100-150 g. body wt.) were obtained from the Animals Breeding Colony of Industrial Toxicology Research Centre (ITRC) two weeks prior to the experiment. Pellet diet was supplied ad libitum and rats had free access to water.

Methods :

Hexachlorocyclohexane treatment :-

Hexachlorocyclohexane (Technical grade HCH) dissolved in peanut oil was administered orally. In single exposure studies

blood was drawn from rat (90-100 g; body weight) in heparinized tube 24 hr after the administration of HCH (100 mg/kg body weight, approximately 1/16th of the LD_{50} values) and then animals were sacrificed. In repeated exposure studies rat (initial weight 90-100 g) were administered 5 mg/kg body weight of HCH. Control rats received peanut oil in an identical manner. Blood was drawn in heparinized tubes at 24 hr after final dose and then the animals were sacrificed.

Erythrocytes and ghost membrane preparation :-

Erythrocytes were prepared by the method of Steck and Kant (1974). Rapid hemolysis was affected by thoroughly mixing 1 ml of packed cells with 40 ml of sodium phosphate (10 mM, pH 8.0) solution. The membranes (ghosts) was pelleted by centrifugation at 12000 xg for 30 minutes.

Extraction and estimation of phosphoinositides from erythrocyte membrane and brain tissue :-

Rats were sacrificed by cervical dislocation and the head were dropped in liquid nitrogen within 5-8 seconds after decapitation. As soon as the frozen heads were thawed the forebrains (cerebra) were removed and used for phosphoinositide extraction as described recently (Subramoniam *et al.*, 1989).

Ghost membranes (separated from 5 ml blood) or the cerebrum (forebrain) were homogenized in 15 ml of chloroform/methanol (1:1 v/v) containing 4 mM calcium chloride in a pestle and mortar. The homogenate was filtered. The filtrate was saved. The residue was washed with 10 ml of

chloroform/methanol (2:1 v/v). The washed residue was homogenized in 15 ml chloroform/methanol (2:1 v/v) containing 0.25% 12 N HCl and 5 mg EDTA under ice cold condition for 1 min in a pestle and mortar. The homogenate was allowed to stand for 30 minutes with occasional stirring and was filtered. The filtrate was shaken well with 0.2 V of 1N HCl. Lower phase was collected and neutralized with 2M ammonium hydroxide. All the phosphoinositides a major portion of phosphatidic acid (PA) and a small portion of phosphatidylinositol (PI) were extracted in this fraction. Samples were evaporated with nitrogen gas and dissolved in minimum quantities of chloroform/methanol (3:1 v/v) and used for thin layer chromatography (TLC).

Phosphatidylinositol (PI), phosphatidyl inositol 4-phosphate (PIP) and phosphatidyl inositol 4,5-bisphosphate (PIP_2) were separated on TLC plates according to the method of Gonzalez-Sastre and Folch (1968) and quantified as described by Subramoniam and Subrahmanyam (1982). A portion of PI extracted in the above filtrate was separated on heat activated silica gel G plates with choroform/methanol/acetic acid (65:15:5 v/v) as a developing solvent and quantified as described by Subramoniam and Subrahmanyam (1982).

Determination of (2-^{3}H)-inositol incorporation into phosphoinositides of erythrocyte membranes :-

Erythrocyte membrane obtained from control or treated rats were incubated in a final volume of 0.5 ml containing 25 mM imidazole-HCl buffer pH 7.0; 5mM $MgCl_2$; 1mM ATP; 1mM EGTA; 0.2 ml ghost and 8 ul of (1.0 uCi/ml (2-^{3}H)-inositol 18.2 Ci/m mole. Amersham) for 1 hr in an agitated water bath at 37°C (Quist,

1982). The incubation was stopped by the addition of an equal volume of ice cold 15% TCA. The percipitate was extracted with 1 ml chloroform/methanol (2:1 v/v) containing 0.5% HCl. Then 0.2 ml of 0.1 M KCl was added and mixed well to separate the phases. The lower organic phase was collected and dried under nitrogen. The phosphoinositides were separated from the lipid extract on silica gel H chromatography plates impregnated with 1.5% potassium oxalate as described (Subramoniam *et al.*, 1989) and appropriate portion of silica gel was scraped off in 10 ml scintillation fluid and radio-activity was determined.

Assay of ATPase :-

Ca^{2+}-Mg^{2+}-ATPase was assayed in ghost membrane as described here. The reaction medium in a final volume of 1.5 ml contained imidazole HCl buffer (135 mM, pH 7.5), $MgCl_2$ (5mM), $CaCl_2$ (0.05 mM). ATP (4mM) and ghost suspension (0.2-0.3 ml). The reaction mixture was incubated for 1 hr at 37°C and the reaction was stopped by the addition of 0.1 ml chilled TCA (50%). The inorganic phosphate (Pi) liberated was estimated by the method of Peterson (1978). Total ATPase activity was measured in the presence of Ca^{2+} and Mg^{2+} in the reaction mixture, while Mg^{2+}-ATPase was measured in the presence of 0.5 mM EDTA. The Ca^{2+}-ATPase activity was determined by the difference between the total and Mg^{2+}-ATPase and was expressed as umol Pi/mg protein/hr. The protein content of the erythrocyte membranes was estimated according to Lowry *et al.*, (1951).

Assay of Acetylcholinesterase activity :-

Acetylcholinesterase was assayed as described by Ellman (1961). The reaction medium in a final volume of 3.17 ml contained phosphate buffer (0.1 M, pH 8.0), acetylthiocholine iodide (0.075 M), 5,5'-Dithiobis (2-Nitrobenzoic acid) (0.01 M), and ghost suspension 20-50 ul. 5,5'-Dithiobis(2-nitrobenzoic acid) (0.01 M) was prepared in phosphate buffer (0.1 M, pH 7.0) and $NaHCo_3$ (179 mM). The absorbance was read at 412 nm. The enzyme activity was expressed in mol/min/mg.

Determination of reduced glutathione :-

Reduced glutathione (GSH) was determined in blood by the modified method of Beutler *et al.*, (1963). 0.2 ml blood was precipitated by 1 ml TCA (10%) followed by addition of 0.8 ml distilled H_2O. Centrifuged at 2000 xg for 10 min. 0.5 ml supernatant was taken to this 2 ml phosphate solution (0.3 M, Na_2HPO_4) and 0.25 ml DTNB were added. Reading was taken at 412 nm. No fading of colour occured for atleast 5 min after the addition of DTNB reagent. After 10 min of standing there was frequently loss of 1-2% of the colour.

Liver lipid peroxidation :-

Lipid peroxidation was estimated in 10% liver homogenates by the method described by Bernheim *et al.*, (1948). Liver homogenate (10%) was prepared in 0.15 M KCl. Liver homogenate (10 ml) was taken in conical flask and placed in metabolic shaker water bath at 37^{o}C. After 0 min and 1 hr 1 ml sample was taken, precipitated with 1 ml 10% TCA, and centrifuged at

1000 xg for 10 minutes. To the supernatant 1 ml thiobarbituric acid (0.67%) was added and mixed thoroughly.

The solution was heated for 10 minutes in boiling water bath. After cooling the absorbance of the sample was read at 535 nm against a blank that contained all the reagent minus the lipid. The malondialdehyde (MDA) concentration of the sample was calculated using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

One dimensional sodium dodecyl sulphate - polyacrylamide gel electrophroesis (SDS-PAGE) of rat erythrocyte membrane :-

The overall approach was that of Laemmli (1970) with several modifications. Gel polymerization followed generally the alterative procedure of Davis (1964). Gel containing 7.5% acrylamide were prepared from a stock solution of 30% by weight of acrylamide and 0.8% by weight of N. N'-bismethylene acrylamide. The final concentration of the gel were as follows: 0.375 M Tris HCl (pH 8.8) and 0.1% SDS. The gel were polymerized chemically by the addition of 0.025% by volume of tetramethyl ethylene diamine (Temed) and ammonium persulphate. The samples (0.2-0.3 ml) contained the final concentrations (final sample buffer): 0.0625 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromophenol blue as dye. The proteins were completely dissociated by immersing the samples for 3 minutes in boiling water, 25 ug to 100 ug protein samples were loaded in the gel well for silver and coomassie brilliant blue R-250 staining respectively. The electrode buffer (pH 8.3) contained 0.025 M Tris, 0.0192 M glycine and 0.1% SDS. Electrophoresis was carried out at 100 volt in the

initial stage till the samples intered the gel then it was increased to 150 volt. The running time under these conditions was about 4 h.

Staining with coomassie brilliant blue R-250 :-

This is the standard method of protein detection.

(i) Staining solution was prepared as follows : 0.1% coomassie brilliant blue R-250 (w/v) in 40% methanol (v/v), 10% acetic acid (v/v). Staining solution was filtered after the dye has dissolved. The staining solution stored at room temperature was reusable.

(ii) The gel was soaked in excess of staining solution for 24 hrs.

(iii) Destained with a large excess of 40% methanol, 10% acetic acid. Destaining solution was changed several times until the background was satisfactorily removed.

The acid alcohol solution used in this procedure did not completely fix proteins in the gel. This led to loss of some low molecular weight proteins during staining and destaining with the dye. Permanent fixation was obtained by incubating the gel in 40% methanol (v/v), 10% trichloroacetic acid (w/v) for 1 h before it was finally immersed in the staining solution.

Silver staining :-

Method developed by Merril and Co-Workers was used for siliver staining. Reaction time varied with the thickness of the gel. Proteins in the gel were fixed in about 400 ml of 40% methanol, 10% acetic acid (v/v) for 30 minutes, followed by twice in 400 ml 10% ethanol, 5% acetic acid (v/v) for 15-30

minutes. The gel was soaked for 3-10 minutes in 200 ml of fresh oxidizer solution (0.0034 M potassium dichromate, 0.0032 N nitric acid), three or four times for 5-10 minutes in 400 ml H_2O, until the yellow colour had been washed out. After this the gel was soaked in 200 ml fresh silver reagent (0.012 silver nitrate) for 15-30 minutes followed by 400 ml H_2O for 1-2 minutes. The gel was then soaked for about 1 minutes in developer solution (0.28 M sodium carbonate, 1.85% paraformaldehyde). The developer was replaced with fresh solution and the development was allowed to continue till satisfactory staining had been obtained. The development was stopped with 5% acetic acid (v/v).

Osmotic fragility

The blood was collected into heparinized vials and centrifuged at 1000 xg to separate out the buffy coat. The RBC were washed repeatedly with normal saline and processed for osmotic fragility studies according to Parpart *et al.*, (1947).

Results

The effect of single and repeated oral administration of HCH on phosphoinositide levels in rat erythrocyte membranes (ghosts) is given in Table-1. The levels of PI, PIP and PIP_2 did not show any change after 2 h of HCH treatment (100 mg/kg body wt.). There was a slight but non significant tendency to decrease in the level of PI, PIP and PIP_2 after 24 h of HCH treatment. Repeated oral administration of HCH to rats at dose of 5 mg/kg body weight (5 days a week) for three months caused the erythrocyte membranes PI, PIP and PIP_2 levels to decline by 16%, 13% and 13% respectively. After six months of HCH treatment (5 mg/kg b.wt.) the levels of PI, PIP and PIP_2 were reduced by 59%, 68% and 37% respectively, as compared with the controls.

The effect of HCH on phosphoinositide levels in rat forebrain (cerebrum) after single and repeated treatment are given in Table-2. There was no change in PI, PIP and PIP_2 levels in cerebrum after 2 h of the single treatment (100 mg/kg

TABLE-1

Effect of hexachlorocyclohexane on phosphoinositides in rat erythrocyte membranes (Ghosts)

	n mol/ml blood		
	PI	PIP	PIP_2
Control	170.4±15	12.4±1.5	23.7±2
Control (3 months)	171.4±13	12.5±1.4	24.1±2
Control (6 months)	169.5±12	12.3±1.5	24.3±2
Hexachlorocyclohexane treated			
Single exposure			
100 mg/kg; 2 h	180.4±10	13.0±3	24.0±4
100 mg/kg; 24 h	155.2±9 (9)	11.8±2 (5)	22.5±3 (5)
Repeated exposure			
5 mg/kg; 3 months	144.9*±17 (16)	10.9*±1.7 (13)	21.0*±3 (13)
5 mg/kg; 6 months	69.2±9 (59)	4.0*±1.6 (67)	14.7*±4 (37)

*P<0.001

Values are means ± S.E. (n=5, in each case)

Values within brackets represent precent decrease of respective control values.

PI : Phosphatidylinositol

PIP : Phosphatidylinositol 4-phosphate

PIP_2 : Phosphatidylinositol 4,5-bisphosphate.

TABLE-2

Effect of hexachlorocyclohexane on phosphoinositides in rat forebrain (cerebrum)

	n mol/g wet cerebrum		
	PI	PIP	PIP_2
Control	2022±100.2	264.0±10.0	395.8±34.0
Control (3 months)	1994± 30.5	260.9±12.5	400±37.8
Control (6 months)	2050±35.0	267.1± 5.8	396.2±39.5
Hexachlorocyclohexane treated			
Single exposure			
100 mg/kg; 2 h	1990±162 (2)	260.1±16 (2)	385.2±25 (3)
100 mg/kg; 24 h	1785±140 (12)	245.7±17 (7)	355.8±31 (10)
Repeated exposure			
5 mg/kg; 3 months	1264*±89 (37)	180.7*±13 (31)	293.3*±29 (27)
5 mg/kg; 6 months	968.4*±84 (53)	149.5*±12 (44)	256.2*±25 (35)

*P<0.001

Values are means ± S.E. (n=5, in each case)

Values within brackets represent percent decrease of respective control values.

PI : Phosphatidylinositol

PIP : Phosphatidylinositol 4-phosphate

PIP_2 : Phosphatidylinositol 4,5-bisphosphate

body wt.). A slight change in phosphoinositide levels was observed at 24 h after the treatment, but it fell short of statistical significance. Repeated exposure of rats to HCH 5 mg/kg body weight (5 days a week) resulted in a marked decrease in the levels of all the three major phosphoinositides. The levels of PI, PIP and PIP_2 were reduced by 37%, 31% and 27% respectively after HCH administration for three months. When the treatment was continued for six months, the levels of PI, PIP and PIP_2 were decreased further (53%, 44% and 35% respectively).

As shown in Table-3 erythrocyte membranes obtained from rats treated with a single dose of HCH (100 mg/kg b.wt.) for 24 h showed a marked increase in the incorporation of (2-^{3}H) inositol into phosphoinositides. There was 260% increase in the (2-^{3}H) inositol incorporation in treated rat ghost PI as compared to that in the control rats, whereas the increase in the incorporation in polyphosphoinositides (PIP + PIP_2) was 178%.

The effect of single and repeated oral administration of HCH on rat erythrocyte membranes ATPase (Ca^{2+}-Mg^{2+}-ATPase) is given in Table -4. The activity of Ca^{2+}-Mg^{2+}-ATPase (membrane bound enzyme) was inhibited at 24 h after the treatment (100 mg/kg body wt.). The overall activity of Ca^{2+}-Mg^{2+}-ATPase was inhibited by 12% whereas the inhibition in Ca^{2+}-ATPase and Mg^{2+}-ATPase were 8% and 12% respectively. Repeated oral administration of HCH to rats at a dose of 5 mg/kg body wt.(5 days a week) for one month caused the erythrocyte membranes Ca^{2+}-Mg^{2+}-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase activities to be

TABLE-3

Effect of HCH exposure on the incorporation of [2-^{3}H] inositol into erythrocyte membrane phosphoinositides

	CPM/mg protein	
	Phosphatidyl-inositol (PI)	Polyphospho-inositides (PIP + PIP_2
Control	23964 ± 476	2543 ± 164
Hexachlorocyclohexane treated (100 mg/kg)	62220* ± 4767 (260)	4529* ± 71 (178)

*$P<0.001$

Values are mean ± S.E.; (n=3, in each case)

Values in parenthesis represent percent increase over the respective control values.

Hexachlorocyclohexane was administered orally (100 mg/kg) and blood was drawn 24 h after the treatment.

PIP : Phosphatidylinositol 4-phosphate

PIP_2 : Phosphatidylinositol 4,5-bisphosphate

TABLE-4

Effect of hexachlorocyclohexane on adenosine triphosphatase (Ca^{2+} Mg^{2+}-ATPase) activity in rat erythrocyte membranes (ghosts).

	μ mol Pi/mg Protein/h		
	$Ca^{2+}Mg^{2+}$-ATPase	Ca^{2+} ATPase	Mg^{2+} ATPase
Control	2.14±0.03	1.50±0.12	0.64±0.05
Hexachlorocyclo-hexane treated			
Single exposure			
100 mg/kg; 24 h	1.88±0.14 (12)	1.38±0.09 (8)	0.50±0.03 (12)
Repeated exposure			
5 mg/kg; 1 month	1.710±0.09 (20)	1.076±0.11 (28)	0.62±0.07 (3)
5mg/kg; 2 months	1.65 ±0.66 (23)	1.012±0.21 (34)	0.54±0.05 (16)

$P<0.002$

Values are mean ± S.E. (n = 5, in each case)

Values within brackects represent percent decrease over respective control values.

inhibited by 20%, 28% and 3% respectively. When the treatment was continued for two months, the activities of Ca^{2+}-Mg^{2+}-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase were inhibited by 23%, 34% and 16% respectively as compared to control of the same experiment.

Effect of HCH on blood glutathione (GSH) level and acetylcholinesterase (AchE) activity in rat erythrocyte membranes (Ghosts) is summarized in Table-5. Repeated oral administration at a dose of 5 mg/kg body weight (5 days a week) caused the blood GSH level to reduce by 14%, 34% and 37% after one, two and three months respectively as compared to control. The activity of erythrocyte membranes acetylcholinesterase remained nearly the same at 24 hr after the treatment (100 mg/kg body wt.) and after one, two and three months of exposure 5 mg/kg body weight (5 days a week) as compared to control rats of the same experiments.

Effect of repeated oral administration of HCH (5 mg/kg body wt., 5 days a week), in liver lipid peroxidation is summarized in Table-6. There was an increase in lipid peroxidation by 18%, 37% and 56% after one, two and three months respectively.

Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) of the rat erythrocyte membrane protein was done after one month of HCH exposure (5 mg/kg body wt) as shown in (Fig. 5a, 5b). But no change in the pattern of erythrocyte membranes protein was observed qualitatively in HCH exposed rats as compared to control rats.

TABLE-5

Effect of hexachlorocyclohexane on blood glutathione (GSH) level and acetylcholinesterase (AchE) activity on rat erythrocyte membranes (Ghosts).

	Blood GSH (mg/100 ml)	AchE activity (mol/min/mg protein)
Control	26.020±0.87	$2.9 \times 10^{-3} \pm 0.2$
Hexachlorocyclo-hexane treated		
5 mg/kg; 1 month	22.309*±0.82 (14)	$2.8 \times 10^{-3} \pm 0.86$ (4)
5 mg/kg; 2 months	17.282*±0.99 (34)	$2.77 \times 10^{-3} \pm 0.77$ (5)
5 mg/kg; 3 months	16.293*±0.74 (37)	$2.76 \times 10^{-3} \pm 0.91$ (5)

*$P < 0.001$; $P < 0.002$

Values are mean ± S.E. (n=4, in each case)

Values within brackets represent percent decrease over respective control values.

TABLE-6

Effect of hexachlorocyclohexane (5 mg/kg b.wt.) on rat liver lipid peroxidation (LPO) after different time of exposure.

	Control	One month	Two months	Three months
Time	nmoles MDA/ mg protein	nmoles MDA/ mg protein	nmoles MDA/ mg protein	nmoles MDA/ mg protein
0 min	1.313±0.43	1.41±0.37 (7)	1.541±0.52 (17)	1.597±0.97 (21)
1 hr	5.572±0.93	6.592±0.68 (18)	7.639±0.89 (37)	8.686±1.12 (56)

$P < 0.002$

Values are mean ± S.E. (n =4, in each case)

Values within brackets indicates percent increase in lipid peroxidation over respective control value

MDA : Malondialdehyde

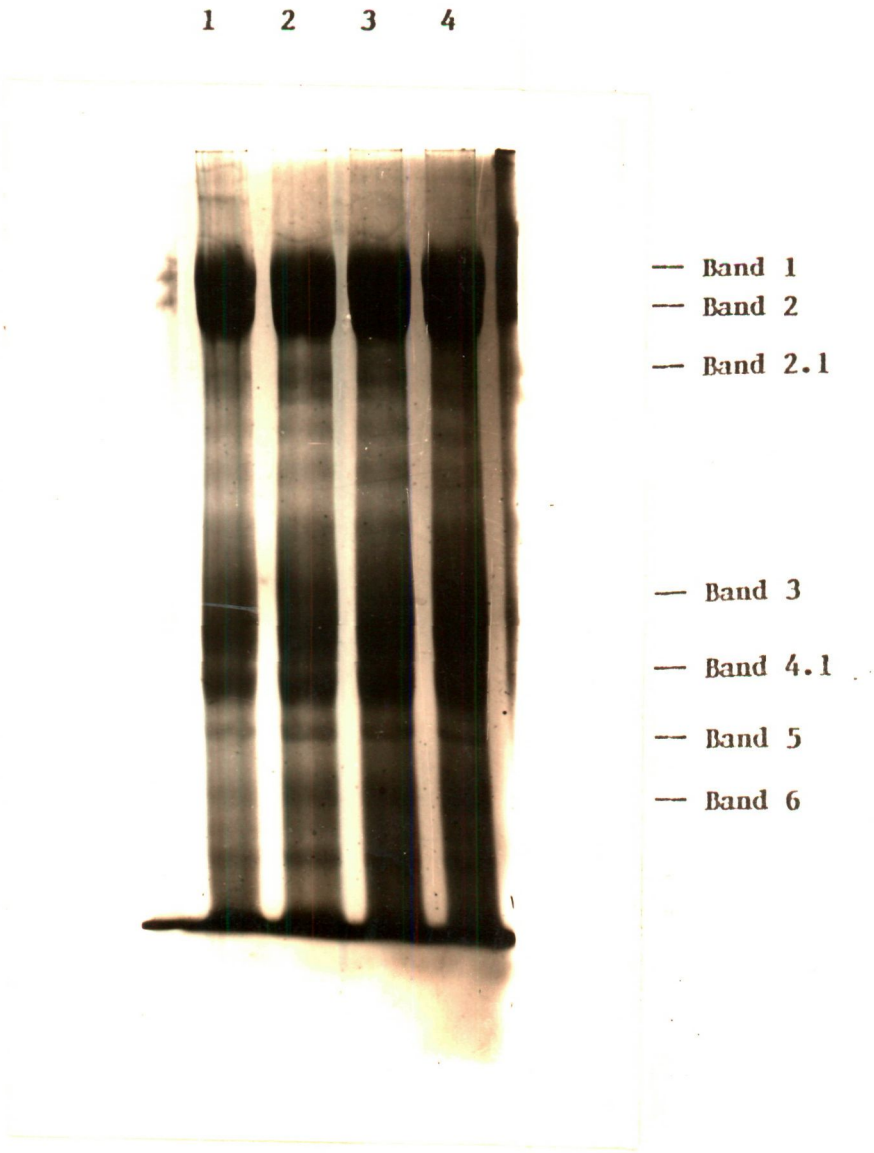


Fig. 5a : SDS-polyacrylamide gel electrophoresis of rat erythrocyte membrane proteins: Lane 1&2: Control, Lane 3&4 after one month of HCH exposure (Coomassie brilliant blue R-250 staining).

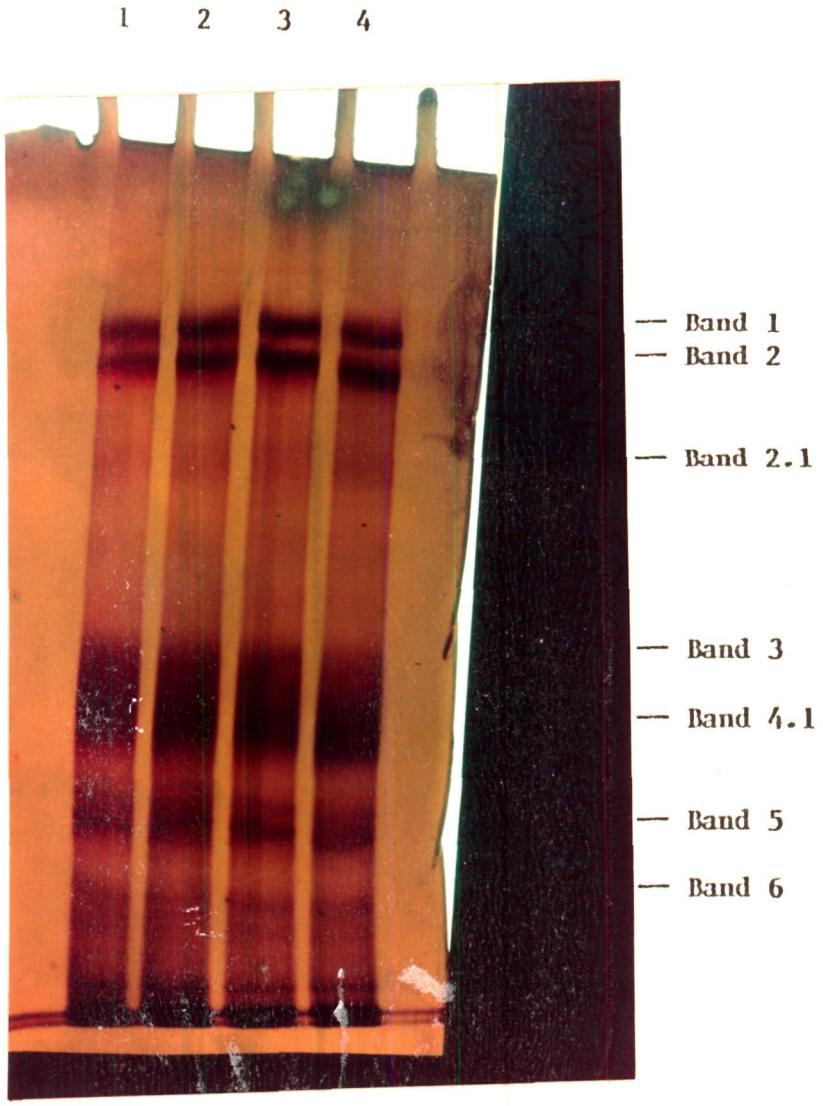


Fig. 5b : SDS-polyacrylamide gel electrophoresis of rat erythrocyte membrane proteins: Lane 1&2: Control, Lane 3&4 after one month of HCH exposure (Silver staining).

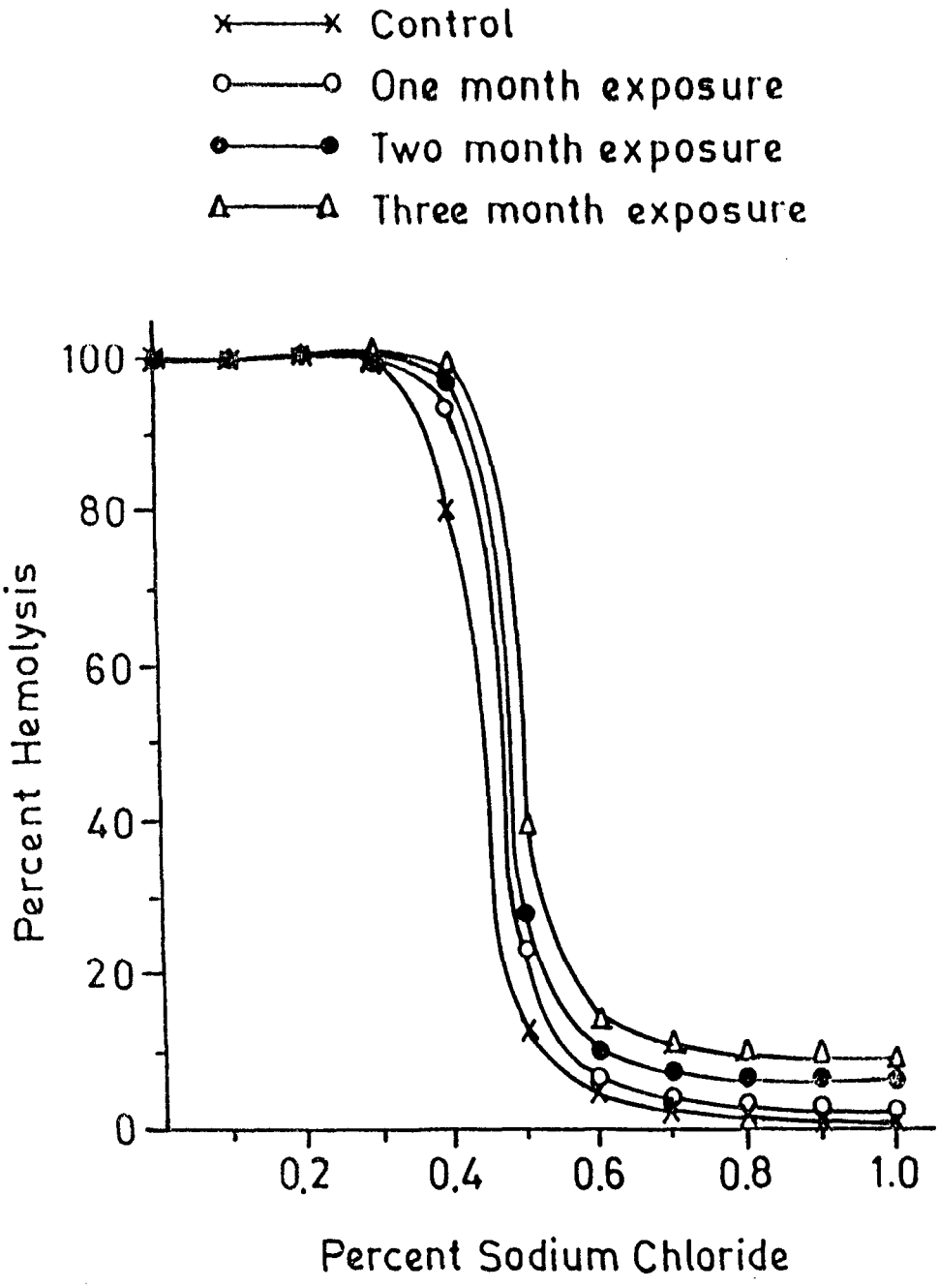


Fig.6. Osmotic fragility curve after HCH exposure in rats in vivo .

Effect of HCH on osmotic fragility of RBC after HCH exposure to rats is shown (Fig. 6). Osmotic fragility as evident from the curve changed after one, two and three months of treatment. There was an over all tendency of the cells to hemolyse as a result of HCH exposure, indicating a fragile nature.

Discussion

Hexachlorocyclohexane is a stable and persistent organochlorine insecticide extensively used in agriculture (Hayes, 1982). The insecticidal action of hexachlorocyclohexane, as well as some of its effects elicited in mammals, appears to involve action on the nervous system, presumably by modulation of acetylcholine release (Doherty, 1979). This effect of HCH has been related to its highly lipophilic nature, which allows the accumulation of the agent in cellular membranes with the consequent perturbation of membrane permeability (Madeira *et al.*, 1985). Oral exposure of rats to HCH for 24 h (100 mg/kg b.wt.) and after three and six months (5 mg/kg b.wt.) markedly reduced (*$P<0.001$) the levels of phosphoinositides in erythrocyte membranes (Table-1) as well as in forebrain (Table-2). It may be suggested from the present study that chronic HCH treatment might adversely affect vital membrane and cell functions modulated by phosphoinositides. Inositol phospholipids play a critical role in translating many hormonal and

neurotransmitter signals at cell surface receptors into appropriate cellular responses. A marked decrease in the levels (Table-2) of phosphoinositides observed in chronically HCH treated rat cerebrum could possibly derange the normal physiological responses brought about by phosphoinositide linked neurotransmitter receptors (Costa, 1990). It is of interest to note that lindane is known to cause behavioural changes (Rivera *et al.*, 1990) and alterations in the levels of PI-linked neurotransmitter (Rivera *et al.*, 1991).

Although the steady state levels of phosphoinositides showed a trands to decrease in erythrocyte membranes obtained from rats, 24 h after treatment with a single dose of HCH (Table-1), the rate of synthesis of phosphoinositides (PI) and polyphosphoinositides ($PIP+PIP_2$) increased as judged from a marked increase in the incorporation of (2-^{3}H)-inositol into phosphoinositides and polyphosphoinositides (Table-3). This indicates that the rate of hydrolysis of phosphoinositides could have been increased more than the increase in the synthesis resulting in a decrease in the level of phosphoinositides. Thus the phosphoinositides turnover and generation of second messengers from phosphoinositides were increased from the treated erythrocyte membranes. Hydrolysis of membrane phosphoinositides to produce water soluble inositol phosphorylated derivatives and diacylglycerol constitute a transmembrane transduction mechanism known to regulate a large assay of cellular processes including neural activity (Michell *et al.*, 1989; Fisher *et al.*, 1992). One of these messengers inositol 1,4,5 triphosphate, is known to play an important role

in the generation of intracellular calcium signals (Berridge and Irvine, 1989). The significant decrease in phosphoinositides observed in long term HCH treated rats could be due to gradual accumulation of HCH or its decomposition product(s). It has been observed that HCH is present in rat erythrocytes (0.18 mg/ml erythrocytes) when the animals were fed 50 mg/kg HCH (commercial, 50% active component) 5 days a week for one month (Agrawal *et al.*, 1992). Even though the messenger systems in signal transduction work under suboptimal saturation levels so that small changes in inositol-phosphates may not be reflected quantitatively in genetic expression, the data on inositide levels in thymocytes, in the presence of mitogen and inhibitor, indicate feedback regulation (Taylor *et al.*, 1984).

The activity of $Ca^{2+}Mg^{2+}$-ATPase of the rat erythrocyte membrane was inhibited after 24 h (100 mg/kg b.wt.), one and two month (5 mg/kg b.wt.) of HCH exposure (Table - 4). This results in ionic imbalance. Although the insecticide does not effect the general membrane fluidity (Madiera *et al.*, 1989), it has been shown to perturb the activity of (membrane bound enzymes) erythrocyte ATPases (Demael *et al.*, 1987). The precise mechanism whereby HCH inhibits membrane-associated enzymes is not clear, but it appears more likely to involve an interaction with hydrophobic domains of the enzymes or their associated annular lipids than to involve a general perturbation of membrane bilayer structure. An interaction of HCH with associated annular lipids of these enzymes could produce a distortion of the three-dimensional structure of the enzymes involved, with resulting decrease in both substrate affinities

and reaction velocities. Changes in enzyme conformation might also occur due to insertion of HCH molecules directly into hydrophobic domains of the proteins.

The activity of acetylcholinesterase (AchE) was also inhibited, but the inhibition was not very marked as shown (Table-5). The exact role of AchE in erythrocyte membranes is still not clear. Giberman *et al.*, (1973) have shown that possibly AchE is envolved in ion transport and permeability in the cells. The toxic effect of organochlorine pesticides are reported to be due to interruptions of energy mobilising and sensory transmitting system of which ATP and ATPase are integral part.

Glutathione (GSH; γ-glutamyl cysteinylglycine) is a major cellular soluble thiol. GSH plays a key role in the detoxification of electrophiles or free radical metabolites of xenobiotics. The detoxification of certain free radical metabolites was shown to be due to the interaction of xenobiotics derived free radicals with glutathione (GSH) forming thiyl radical (GS$^{\cdot}$) (Ross, 1988). The contribution of erythrocytes to the protection against reactive metabolites depends on the ability of metabolites to pass the erythrocyte membranes (Fazi *et al.*, 1991). There was a decrease in the level of GSH after HCH exposure (Table-5). The decrease in blood free -SH group in HCH treated rats is due to GSH depletion through oxidation of free radicals. The results support the view that GSH plays an important role in protecting the cell against oxidative attack by HCH. The GSH concentration in erythrocytes is found to decrease after exposure *in vivo*, to

exogenous oxidants (Trotta *et al.*, 1982). Although possible leakage of GSH from erythrocytes or breakdown of its primary sulphide links with membrane constituents or globin could also lead to lower GSH levels, considerable evidence suggests a role of the thiol in biotransformation. It has been reported that a high proportion of the metabolism of HCH involves conjugation with GSH as a final step (Noack and Portig, 1973).

Effect of HCH on liver lipid peroxidation at different time interval is shown in (Table-6). There was a marked increase in liver lipid peroxidation after HCH treatment. An enchancement in free radical generation, a diminution in the cellular protective defenses, or both can stimulate lipid peroxidation over basal values, leading to wide spread membrane changes (Kappus, 1985; Videla *et al.*, 1988; Comporti, 1985). Lindane elicited a significant dose-dependent increase in TBAR substances by liver homogenates (Junqueira *et al.*, 1986; Goel *et al.*, 1988). Although the changes in liver lipid peroxidation by lindane treatment could be related to the changes in microsomal $O_2^{-\cdot}$ generation, the participation of lindane derived free radical(s) that could be produced by cytochrome P-450 system (Jones *et al.*, 1985). It is sugested that the decrease in the activity of superoxide dismutase and catalase could possibly contribute to excerbate lipid peroxidation, in addition to the enhanced capacity of liver to generate $O_2^{-\cdot}$, observed following lindane treatment (Junqueira *et al.*, 1986).

No qualitative changes in the electrophoretic pattern was observed in the erythrocyte membrane proteins of rat after HCH treatment for one month (Fig. 5a, 5b). As shown from osmotic

fragility curve (Fig. 6). RBC became more fragile and hemolyzed, after one, two and three months of HCH exposure.

The above studies suggest that a relationship may exist between different parameters of rat erythrocytes during delayed toxicity of HCH leading to oxidative membrane damage. Blocking of membrane enzymes such as ATPase and AchE can also cause ionic imbalances resulting in membrane fragility. The results on the inositide levels may be indicative of signal transduction changes in HCH toxicity. The above data suggest that the long term toxicological effects of HCH involve alterations in the properties of cell membranes and cell functions.

Bibliography

Agrawal, D., Sultana, P., and Gupta, G.S.D. (1991). Oxidative damage and Changes in the glutathione redox system in erythrocytes from rats treated with hexachlorocyclohexane. Food. Chem. Toxicol. **29**, 459-462.

Agrawal, D., Sultana, P., Gupta, G.S.D., Gopal, K., Khanna, R.N. and Anand, M. (1992). Effect of hexachlorocyclohexane on biochemical parameters of rats on a protein deficient diet. Toxicol. Environ. Chem. **35**, 109-114.

Agrawal, D., Sultana, P., and Ray, P.K. (1989). Effect of pesticides on red blood cells *in vitro*. Ind. Vet. Med. J. **13**(1), 14-20.

Albono, E., Lott, K.A.K., Slater, T.F., Steer, A., Symons, M.C.R., and Tomasi, A. (1982). Spin trapping studies on the free radical products formed by metabolic activation of carbon tetrachloride in rat liver microsomal fractions isolated hepatocytes an *in vivo* in the rat. Biochem. J., **204**, 593-603.

Allan, D. and Michell, R.H. (1975). Accumulation of 1,2-diacylglycerol in the plasma membrane may lead to echinocyte transformation of erythrocytes. Nature **258**, 348-349.

Allan, D. and Thomas, P. (1981) Ca^{2+} induced biochemical changes in human erythrocytes and their relation to microvesiculation. Biochem. J. **198**, 433-440.

Anand, M., Khanna, R.N., Gopal, K., Gupta, G.S.D. and Sur, R.N. (1980). Effect of endosulfan on bioelectrical activity of brain in rats. Vet. Hum. Toxicol. **22** (6), 385-387.

Baker, M.T., Nelson, R.M. and Dyke, R.A.V. (1985). The formation of chlorobenzene and benzene by the reductive metabolism of lindane in rat liver microsomes. Archives of Biochemistry and Biophysics **236**, 506-514.

Baumann, K., Angerer, J., Heinrich, P. and Lehnert, G. (1980). Occupational exposure to hexachlorocyclohexane. I. Body burden of hexachlorocyclohexane isomers. Int. Arch. Occup. Environ. Hlth., **47**(2), 119-127.

Bernheim, F., Bernheim, M.L.C. and Wilbur, K.M. (1948). The reaction between thiobarbituric acid and the oxidation products of certain lipids. J. Biol. Chem., **174**, 257-264.

Berridge, M.J. (1987). Inositol triphosphate and diacylglycerol : Two interacting second messangers. Ann. Rev. Biochem. 56, 159-193.

Berridge, M.J. and Irvine R.F. (1989). Inositol phosphate and cell signalling. Nature, **341**, 197-205.

Beutler, E., Duron, O. and Kelly, B.M. (1963). Improved method for determination of blood glutathione. J. Lab. Clin. Med. **61**, 882-888.

Burchfiel, J.F., Duffy, F.H. and Sim, V.M. (1979). Persistent effects of sarin and dieldrin upon the primate electroencephalogram. Toxicol. Appl. Pharmacol., **35**, 365-379.

Bus, J.S., Aust, S.D., and Gibson, J.E. (1976). Paraquat toxicity: proposed mechanism of action involving lipid peroxidation. Environ. Hlth. Perspect. **16**, 139-146.

Carter, J.R., Jr. (1973). Role of sulfhydryl groups in erythrocyte membrane structure. Biochemistry, **12**, 171-176.

Chadwick, R.W. and Copeland, M.F. (1985). Investigation of HCB as a metabolite from female rats treated daily for six days with lindane. J. Anal. Toxicol. **9**, 262-266.

Chance, B., Sies, H. and Boveries, A. (1979). Hydroperoxide metabolism in mammalian organs. Phys. Rev. **59**, 527-605.

Choquette, D.C., Hakin, G., Filoteo, A.G., Plishker, G.A., Bostwick, J.R. and Penniston, J.T. (1984). Regulation of plasma membrane Ca^{2+} ATPase by lipids of the phosphatidylinositol cycle. Biochem. Biophys. Res. Commun. **125** 908-915.

Comporti, M. (1985). Lipid peroxidation and cellular damage in toxic liver injury. Lab. Invest. **53**, 599-623.

Costa, L.G. (1990). The phosphoinositide/protein kinase C system as a potential target for neurotoxicity. Pharmacol. Res. **22**, 393-408.

Daniel, C.V., Agrawal, S. and Agrawal, S.S. (1986). Human red blood cell membrane damage by endosulfan. Toxicol. Lett. **32**, 113-118.

Davies, K.J.A., Quintanitha, A.T., Brooks, G.A. and Packer, L. (1982). Free radicals and tissue damage produced by excercise. Biochem. Biophys. Res. Commun. **107**, 1198-1205.

Davis, B.J. (1964). Disc electrophoresis-II method and application to human serum proteins. Ann. N.Y. Acad. Sci. **121**, 404-427.

Demael, A., Lepot, D., Cossarine-Dunier, M., Monod, G. (1987) I. Effect of gamma hexachlorocyclohexane (lindane) on carp (cyrpinus caprio) II. Effects of chronic intoxication on blood liver enzymes, and muscle plasmic membrane. Ecotoxicol. Environ. Safety **13**, 346-351.

Dohety, J.D. (1979). Insecticide affecting ion transport. Pharmacol. Ther. **7**, 123-151.

Ellman, G.L., Courtney, K.D., Andres, V. and Featherstone, R. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology, **7**, 88-95.

Fazi, A., Mancini, U., Piatti, E., Accorsi, A. and Magnmi, M. (1991). Human red blood cells as bioreactors for the inactivation of harmful xenobiotics. Biotechnol. Appl. Biochem. **14**, 60-68.

Fisher, D.B. and Mueller, G.C. (1971). Gamma hexachlorocyclohexane inhibits the inactivation of lymphocyte growth by phytohemagglutinin. Biochem. Pharmacol. **20**, 2515-2518.

Fisher, S.K., Heacock, A.M. and Agranoff, B.W. (1992). Inositol lipids and signal transduction in the nervous system: An update, J. Neurochem. **58**, 18-38.

Fitzloff, J.F. and Pan, J.C. (1984). Epoxidation of the lindane metabolite, gamma-PCCH, by human and rat liver microsomes. Xenobiotica **14**, 599-604.

Fitzloff, J.F., Portig, J. and Stein, K. (1982). Lindane metabolism by human and rat liver microsomes. Xenobiotica **12**, 197-202.

Fowler, V.M. (1986). An actomyosin contraction mechanism for erythrocyte shape transofmration. J. Cell Biochem. **31**, 1-9.

Fukami, K., Furuhasti, K., Inagaki, M., Endo, T., Hatano, S. and Takenawa, T. (1992). Requirement of phosphatidylinositol 4,5-bisphosphate for α-actinin function. Nature. **359**, 150-152.

Gabrylak, T. and Klekot, J. (1985). The effect of paraquat on the peroxide metabolizing enzymes in erythrocyte of fresh water fish species. Comp. Biochem. Physiol. **81**(2), 415-418.

Gendel, L.J., Likhacheva, N.I., Bogonatov, B.N. and Ponasenko, O.M. (1984). Changes in erythrocyte shape under the effect of pesticide-sodium pentafluorophenolate (the scanning electron microscopy method) DOLK Akad. Nauk. SSSR. **227**(2), 493-496.

Gillette, J.R., Mitchell, J.R. and Brodie, B.B. (1974) Biochemical mechanisms of drug toxicity. Annu. Rev. Pharmacol. **14**, 271-288.

Giberman, E., Silman, J. and Edery, H. (1973). Effect of cholinesterase inhibitors on active potassium influx in monkey erythrocytes. Biochemical pharmacology **22**, 271-273.

Goel, M.R., Shara, M.A. and Stochs, S.J. (1988). Induction of lipid peroxidation by hexachlorocyclohexane, dieldrin, TCDD, Carbon tetrachloride, and hexachlorobenzene in rats. Bull. Environ. Contam. Toxicol. **40**, 255-262.

Gonzalez-Sastre and Folch, J. (1968). Thin layer chromatography of phosphoinositides. J. Lipid Res. **9**, 532-533.

Hayes, Jr., W.J. Pesticides studied in Man, Williams and Wilkins, 428, Baltimore, USA, 1982, p. 211.

Heinvetter, D., Lewerenz, H.J., Plass, R., and Macholz, R. (1985). Einfluss von lindane and lindane metabolites auf mikrosomale and mitochondriale ATPasen *in vitro*. J. Environ. Sci. Hlth. **B20**, 539-558.

Hoffmann, R., Erzberger, P., Frank, W. and Ristow, H.J. (1980). Increased phosphatidylinositol synthesis in rat embryo fibroblasts after growth stimulation and its inhibition by 6-hexachlorocyclohexane. BBA. **618**, 282-292.

Hokin, L.E. (1985). Receptors and phosphoinositide generated second messangers. Ann. Rev. Biochem. **54**, 205-236.

Hokin, M.R. and Brown, D.F. (1969). Inhibition by -hexachlorocyclohexane of acetylcholine stimulated phosphatidylinositol synthesis in cerebral cortex slices and of phosphatidic acid inositol transferase in cerebral cortex particulate fractions. J. Neurochem. **16**, 473-483.

Hulth, L., Hoglund, L., Bergman, A. and Moller, L. (1978). Convulsive properties of lindane, lindane metabolites of the lindane isomer α-hexachlorocyclohexane; effects on the convulsive threshold for pentylenetetrazol and the brain content of γ-aminobutyric acid (GABA) in the mouse. Toxicol. Appl. Pharmacol. **46**, 101-108.

Irvine, R.F., Dawson, R.M.C. and Freinkel, N. (1982). In contemporary metabolism (Freinkel, N., ed.) Vol. **2**, pp. 301-342, Plenum Medical Book Co., New York.

Jones, O.T., Froud, R.J. and Lee, A.G. (1985). Interaction hexachlorocyclohexane with the (Ca^{2+}-Mg^{2+}) ATPase from sarcoplasmic reticulum. BBA **812**, 740-751.

Junqueira, V.B.C., Kiyoko Somizu, Videla Luis, A. and Barros, S.B.M. (1986). Dose-dependent study of the effects of acute lindane administration of rat liver superoxide anion production. Antioxidant enzyme activities and lipid peroxidation. Toxicology, **41**, 193-204.

Kappus, H. (1985). Lipid peroxidation : mechanism analysis, enzymology and biological relevance. In sies, H. ed. Oxidative stress. London Academic Press, 273-310.

Kelloqq, E.W. and Fridovich, I. (1977). Liponome oxidation and erythrocyte lysis by enzymically generated superoxide and hydrogen peroxide. J. Biol. Chem. **252**, 6721-6728.

Kobayashi, Y., Okahata, S. and Utsuri, T. (1979). Hemolysin of human erythrocytes by paraquat in relation to superoxide dismutase activity. Biochem. Biophys. Res. Commun. **91**, 1288-1294.

Kumar S.S., Sikka, H.C., Saxena, J. and Zweig, G. (1975). Membrane damage in human erythrocytes caused by captan and captafol. Pestic. Biochem. Physiol. **5**, 338-347.

Kuo, C.H. and Hook, J.B. (1982). Depletion of renal glutathione content and nephrotoxicity of cephaloidine in rabbits, rats and mice. Toxicol. Appl. Pharmacol. **63**, 292-302.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680-685.

Lowry, O.H., Rosebrough, N.J., Farr, W.L. and Randall, R.J. (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem., **193**, 265-275.

Madeira, A.M.C. and Madeira, V.M.C. (1985). Partition of lindane in synthetic and native membranes. Biochem. Biophys. Acta. **820**, 165-172.

Madeira, A.M.C. and Madeira, V.M.C. (1989). Membrane fluidity as affected by the insecticide lindane. Biochem. Biophys. Acta. **982**, 161-166.

McCay, P.B., Lai, E.K., Lee Poyer, J., Du Bose, C.M. and Janzen, E.G. (1984). Oxygen and carbon centered free radical formation during carbon tetrachloride metabolism. Observation of lipid radicals *in vivo* and *in vitro*. J. Biol. Chem. **259**, 2135-2143.

McNamara, B.P. and Krop, S. (1948) Observation of the pharmacology of the isomers of hexachlorocyclohexane. J. Pharmacol. Exp. Ther. **92**, 140-146.

Michell, R.H. (1975). Inositol phospholipids and cell surface receptor function. BBA, **415**, 81-147.

Michell, R.H., Drummond, A.H., Downes, C.P., eds. (1989). Inositol lipids in cell signalling. London, Academic Press.

Misra, D., Khanna, R.N., Anand, M. and Gopal, K. (1982). Interaction of endosulfan with erythrocyte membrane. J. Adv. Zool. **3**, 135-141.

Nigam, S.K., Karnik, A.B., Majumdar, S.K., Visweswerіah, K., Raju, G.S., Bai, K.M., Lakkad, B.C., Thakore and Chatterje, B.B. (1986). Serum hexachlorocyclohexane residues in workers engaged at a HCH manufacturing plant. Int. Arch. Occup. Environ. Hlth., **57**, 315-320.

Nishihara, Y. and Utlsumi, K. (1983). Diminished osmotic fragility and shape alteration of human erythrocytes following the treatment with DDT. Cell. Mol. Biol **29**, 103-111.

Noack, G. and Portig, J. (1973). Biodegradation of alpha HCH III. Decrease in liver non protein thiols after intragastric application of the drug. NaunynSchmiedebergs Archiv fur Experimentale Pathologic und Pharmakologic **280**, 183-189.

Parpart, A.K., Lorenz, P.B., Parpart, E.R., Gregg, J.R. and Chase, A.M. (1947). The osmotic resistance (fragility) of human red cells. J. Clin. Invest. **26**, 126-129.

Peterson, G.L. (1978) A simplified method for analysis of inorganic phosphate in the presence of interfering substances. Anal. Biochem. **84**, 164-172.

Quist, E.E. (1982). Polyphosphoinositides synthesis in rabbit erythrocyte membranes. Arch. Biochem. Biophys. **219**, 58-64.

Ruber, M.D. (1979). Carcinogenicity of lindane. Environ. Res. **19**, 460-481.

Rivera, S., Samfeliu, C. and Rodriguez-Farre, E. (1990). Behavioural changes induced in developing rats by an early postnatal exposure to lindane. Neurotoxicol. Teratol. **12**, 591-596.

Rivera, S., Samfeliu, C., Sunol., C. and Rodriguez-Farre, E. (1991). Regional effects on the cerebral concentration of non-adrenaline, serotonin and dopamine in suckling rats after a single dose of lindane. Toxicol. **69**, 43-64.

Ross, D. (1988). Glutathione, free radicals and chemotherapeutic agents mechanism of free-radical induced toxicity and glutathione dependent protection. Pharmac. Ther. **37**, 231-249.

Sasinovich, L.M., Alckhina, S.M. and Odentsova, I.L. (1974). Course of toxic hepatitis developing due to the prolonged effects of hexachlorocylcohexane preparation. Vrach. Delo. **10**, 133-135.

Schatzman, H.J. (1973). Dependence on calcium concentration and stoichiometry of the Ca pump in human red cells. J. Physiol. **235**, 551-569.

Sikka, H.C., Schwartzel, E.H., Saxena, J., Lynch, R.S. and Zweig, G. (1975). Action of dichlone on the human erythrocytes membrane. Environ. Qurl. Saf. Supp. **3**, 507-512.

Sikka, H.C., Schwartzel, E.H., Saxena, J. and Zweig, G. (1974). Interaction of dichlone with human erythrocytes. 1. Changes in cell permeability. Chem. Biol. Interact. **9**, 261-272.

Stark, L.G., Joy, R.M. and Albertson, T.E. (1983). The persistence of kindled amygdaloid seizures in rats exposed to lindane. Neurotoxicology, **4**, 221-226.

Steck, T.L. and Kant, J.A. (1974) Preparation of impermeable ghosts and inside-out vesicles from human erythrocyte membranes. Methods Enzymol. **31**, 172-180.

Subramoniam, A. (1988). The phosphoinositides in transmembrane signalling. J. Sci. Ind. Res. **47**, 587-601.

Subramoniam, A., Goel, S.K., Pandya, K.P. and Seth, P.K. (1989). Influence of trichloroethylene treatment on phosphoinositides in rat brain. Toxicol. Lett., **49**, 55-60.

Subramoniam, A. and Subrahmanyam, D. (1982). Light induced changes in the phospholipid composition of **Micobacterium smegmatis** ATCC 607. J. Gen. Microbiol. **128**, 419-421.

Simplico, P.D., Dolara, P. and Lodovivi, M. (1984). Blood glutathione as a measure of exposure to toxic compounds. J. Appl. Toxicol., **4**, 227-229.

Taylor, M.V., Metcalfe, J.C., Robin Hesketh, T., Smith, G.A. and Moore, J.P. (1984). Mitogen increase phosphorylation of phosphoinositides in thymocytes. Nature, **312**, 462-465.

Trotta, R.J., Sulhivan, S.G. and Sten, A. (1982). Lipid peroxidation and hemoglobin degradation in red blood cells exposed to T. Butyl-hydroperoxide. Biochemical Journal, **204**, 405-415.

Videla, L.A., Fernandez, V. (1988). Biochemical aspects of cellular oxidative stress. Arch. Biol. Med. Exp. **21**, 85-92.

Vu, N., Chepko, G. and Zelenka, P. 91983). Decreased turnover of phosphatidylinositol accompanies *in vitro* differentiation of embryonic chicken lens epithelial cells into lens fibers. BBA. **750**, 105-111.

Weitzman, S.A. and Weitberg, A.B. (1985). Asbestos catalyzed lipid peroxidation and its inhibition by desferroxamines. Biochem. J., **225**, 259-262.

Wolff, S.P., Garner, A. and Dean, R.T. (1986). Free radicals lipid and protein degradation. Trends Biochem. Sci. **11**, 27-31.

Yvelin, J.M., Yvelin, C. and Livermant, M.C. (1984). Uptake of (U-^{14}C) lindane (γ-hexachlorocyclohexane) following exposure of isolated hepatocytes for short intervals of time. Pestic. Biochem. Physiol. **22**, 349-359.

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Influence of hexachlorocyclohexane on phosphoinositides in rat erythrocyte membranes and brain

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Abstract

Single exposure of rats to hexachlorocyclohexane (100 mg/kg) did not cause any significant change in phosphoinositide levels in rat erythrocyte membrane and cerebrum (fore brain) 2 or 24 h after exposure. However, the phosphoinositide turnover and generation of second messengers from phosphoinositides were increased in the treated erythrocyte membranes as judged from a marked increase in the incorporation of [2-^{3}H]inositol into phosphoinositides 24 h after the treatment. A significant decrease in phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2) was observed in the erythrocyte membrane and cerebrum of rats repeatedly exposed to the pesticide for 3 or 6 months. This drastic reduction in phosphoinositide levels suggests adverse effects on vital membrane and cell functions modulated by phosphoinositides.

Keywords: Hexachlorocyclohexane; Phosphoinositides; Erythrocyte membrane; Cerebrum; Rat

1. Introduction

Hexachlorocyclohexane (HCH) is a stable and persistent organochlorine insecticide extensively used in agriculture (Hayes, 1982). It is known to cause neurological and metabolic disorders (Hayes, 1982). The insecticide lindane (r-isomer of HCH) has been reported to inhibit phytohemagglutinin stimulated phosphatidylinositol turnover (Fisher and Mueller, 1971) and to block acetylcholine stimulated phosphatidylinositol synthesis in cerebral cortex slices (Hokin and Brown, 1969). The phosphoinositide-derived second messengers transduce the signals of many hormones, growth factors and neurotransmitters into appropriate cellular responses (Hokin, 1985; Berridge, 1987; Subramoniam, 1988). Furthermore, phosphoinositides are involved in membrane transport and microfilament dynamics (Subramoniam, 1988; Michell, 1975; Fukami et al., 1992). Many cell surface proteins are anchored to the membrane through phosphatidylinositol (Fisher et al., 1992). The polyphosphoinositides located in the inner leaflet of plasma membranes have a very fast turnover rate (Michell, 1975; Hokin, 1985) and their levels in the cells could be an index of cell as well

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as membrane function. Earlier studies from this laboratory showed that the structure and certain functions of the erythrocyte membrane undergo significant changes as a consequence of HCH exposure (Agrawal et al., 1989; 1991), raising the possibility of the involvement of the phosphoinositide system. Therefore, the present studies were undertaken to examine the steady state levels and/or rate of formation of phosphoinositides in the erythrocyte membrane and brain of hexachlorocyclohexane challenged rats.

2. Materials and methods

2.1. Animals

Female albino rats (weighing 90–100 g) of the Industrial Toxicology Research Centre animal breeding colony were used in this study and were kept on an ad-libitum pellet diet and water under standard laboratory conditions.

2.2. Hexachlorocyclohexane treatment

Hexachlorocyclohexane (Technical grade HCH, Swaroop Chemicals, Lucknow, India) dissolved in peanut oil was administered orally. In single exposure studies, blood was drawn from the rats (90–100 g weight) in heparinized tubes 2 or 24 h after the administration of HCH (100 mg/kg body wt; approx. 1/16th of LD_{50} values) and then animals were killed. In repeated exposure studies rats (initial weight 90–100 g) were administered 5 mg/kg body weight HCH daily (5 days a week) for 3 and 6 months. Control rats received peanut oil in an identical manner. Blood was drawn in heparinized tubes at 20 h after the final dose, and then the animals were killed.

2.3. Erythrocytes and ghost membrane preparation

Erythrocytes were prepared by the method of Steck and Kant (1974). Rapid hemolysis was effected by thoroughly mixing 1 ml of packed cells with 40 ml of a sodium phosphate (10 mM, pH 8.0) solution. The membranes (ghost) were pelleted by centrifugation at 12 000 × *g* for 30 min.

2.4. Extraction and estimation of phosphoinositides from erythrocyte membranes and brain tissue

Rats were killed by cervical dislocation and their heads were dropped into liquid nitrogen within 5–8 s after decapitation. As soon as the frozen heads were thawed the forebrains (cerebra) were removed and used for phosphoinositide extraction as described recently (Subramoniam et al., 1989).

Ghost membranes (separated from 5 ml blood) or the cerebrum (forebrain) were homogenized in 15 ml of chloroform/methanol (1:1, v/v) containing 4 mM calcium chloride in a pestle and mortar. The homogenate was filtered and was then saved. The residue was washed with 10 ml chloroform/methanol (2:1, v/v). The washed residue was homogenized in 15 ml chloroform-methanol (2:1, v/v) containing 0.25% 12 M HCl and 5 mg EDTA under ice-cold conditions for 1 min in a pestle and mortar. The homogenate was allowed to stand for 30 min with occasional stirring and was filtered. The filtrate was shaken well with 0.2 vols. of 1 N HCl. Lower phase was collected and neutralized with 2 M ammonium hydroxide. All the phosphoinositides, a major portion of phosphatidic acid (PA) and a small portion of PI were extracted in this fraction. Samples were evaporated with nitrogen gas and dissolved in minimum quantities of chloroform/methanol (3:1, v/v) and used for thin-layer chromatography (TLC).

PI, PIP and PIP_2 were separated on TLC plates according to the method of Gonzalez-Sastre and Folch (1968) and quantified as described by Subramoniam and Subrahmanyam (1982). A portion of PI extracted in the above filtrate was separated on heat activated silica gel G plates with chloroform/methanol/acetic acid (65:15:5, v/v) as a developing solvent and quantified as described elsewhere (1982).

2.5. Determination of [2-^{3}H]inositol incorporation into phosphoinositides of erythrocyte membranes

Erythrocyte membranes obtained from control or treated rats were incubated in a final volume of 0.5 ml containing 25 mM imidazole-HCl buffer (pH 7.0); 5 mM $MgCl_2$; 1 mM ATP; 1 mM EGTA; 0.2 ml ghost and 8 μl (1.0 μCi/ml [2-^{3}H]inositol 18.2 Ci/mmol. Amersham) for 1 h in an agitated water bath at 37°C (Quist, 1982). The incubation was stopped by the addition of an equal volume of ice-cold 15% TCA. The precipitate was collected by centrifugation and extracted with 1 ml chloroform-methanol (2:1, v/v)

containing 0.5% HCl. Then 0.2 ml of 0.1 M KCl was added and mixed well to separate the phases. The lower organic phase was collected and dried under nitrogen. The phosphoinositides were separated from the lipid extract on silica gel H chromatographic plates impregnated with 1.5% potassium oxalate as described (Subramoniam et al., 1989) and an appropriate portion of silica gel was scraped off in 10 ml scintillation fluid and radioactivity was determined.

3. Results

The effect of single and repeated oral administration of HCH on phosphoinositide levels in rat erythrocyte membranes (ghosts) is given in Table 1. The levels of PI, PIP and PIP_2 did not show any change after 2 h of HCH treatment (100 mg/kg body wt). There was a slight but not significant tendency to decrease the levels after 24 h of the treatment.

Repeated oral administration of HCH to rats at a dose of 5 mg/kg body weight (5 days a week) for 3 months caused the erythrocyte membrane PI, PIP and PIP_2 levels to decline by 16%, 13% and 13%, respectively. After 6 months of HCH treatment to rats (5 mg kg body wt) the levels of PI, PIP and PIP_2 were reduced by 59%, 67% and 37%, respectively (compared to controls of the same experiment).

The effects of HCH on phosphoinositide levels in rat forebrain (cerebrum) after single and repeated treatment are given in Table 2. There was no change in PI, PIP and PIP_2 levels in cerebrum after 2 h of the single treatment (100 mg/kg body wt). A slight change in the phosphoinositide levels was observed at 24 h after the treatment, but it fell short of statistical significance. Repeated exposure of rats to HCH (5 mg kg body wt, 5 days a week) resulted in a marked decrease in the levels of all the three major phosphoinositides. The levels of PI, PIP and PIP_2 were reduced by 37%, 31% and 27%, respectively, after HCH administration for 3 months. When the treatment was continued for 6 months the levels of PI, PIP and PIP_2 were decreased further (53%, 44% and 35%, respectively).

Table 1
Effect of hexachlorocyclohexane on phosphoinositides in rat erythrocyte membranes (Ghosts)

	nmol/ml blood		
	PI	PIP	PIP_2
Control	170.4 ± 15	12.4 ± 1.5	23.7 ± 2
Control (3 months)	171.4 ± 13	12.5 ± 1.4	24.1 ± 2
Control (6 months)	169.5 ± 12	12.3 ± 1.5	24.3 ± 2
Hexachlorocyclohexane treated			
Single exposure			
100 mg kg; 2 h	180.4 ± 10	13.0 ± 3	24.0 ± 4
100 mg/kg; 24 h	155.2 ± 9	11.8 ± 2	22.5 ± 3
	(9)	(5)	(5)
Repeated exposure			
5 mg/kg, 3 months	144.9 ± 17	10.9 ± 1.7	21.0 ± 3
	(16)	(13)	(13)
5 mg/kg; 6 months	69.2* ± 9	4.0* ± 1.6	14.7 * 4
	(59)	(67)	(37)

*$P < 0.001$.
Values are means ± S.E. ($n = 5$, in each case).
Values within brackets represent % decrease of respective control values.
Abbreviations: PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate
Details of treatment are given in Materials and Methods.

Table 2
Effect of hexachlorocyclohexane on phosphoinositides in rat forebrain (cerebrum)

	nmol/g wet cerebrum		
	PI	PIP	PIP_2
Control	2022 ± 100.2	264.0 ± 10.0	395.8 ± 34.0
Control (3 months)	1995 ± 30.5	260.9 ± 12.5	400.5 ± 37.8
Control (6 months)	2050 ± 35.0	267.1 ± 5.8	396.2 ± 39.5
Hexachlorocyclohexane treated			
Single exposure			
100 mg/kg; 2 h	1990 ± 162	260.1 ± 16	385.2 ± 25
	(2)	(2)	(3)
100 mg/kg; 24 h	1785 ± 140	245.7 ± 17	355.8 ± 31
	(12)	(7)	(10)
Repeated exposure			
5 mg/kg; 3 months	1264* ± 89	180.7* ± 13	293.3* ± 29
	(37)	(31)	(27)
5 mg/kg; 6 months	968.4* ± 84	149.5* ± 12	256.2* ± 25
	(53)	(44)	(35)

*$P < 0.001$.
Values are means ± S.E. ($n = 5$, in each case).
Values within brackets represent % decrease of respective control values.
Abbreviations. PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate.
Details of treatment are given in Materials and Methods.

Table 3
Effect of HCH exposure on the incorporation of [2-^{3}H]inositol into erythrocyte membrane phosphoinositides

	CPM/mg protein	
	Phosphatidyl-inositol (PI)	Polyphosphoinositi-des (PIP + PIP_2)
Control	23 964 ± 476	2543 ± 164
Hexachloro-cyclohexane treated (100 mg/kg)	62 220 ± 4767* (260)	4529 ± 71* (178)

Values are mean ± S.E.; $n = 3$; *$P < 0.001$.
Values in parentheses represent % increase over the respective control values.
Hexachlorocyclohexane was administered orally (100 mg/kg) and blood was drawn 24 h after the treatment.
Abbreviations: PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate.

As shown in Table 3 erythrocyte membranes obtained from rats treated with a single dose of HCH (100 mg/kg body wt) for 24 h showed a marked increase in the incorporation of [2-^{3}H]inositol into phosphoinositides. There was a 260% increase in the [2-^{3}H]inositol incorporation in treated rat ghost PI as compared to that in control rats, whereas the increase in the incorporation in polyphosphoinositide was 178%.

4. Discussion

Oral exposure of rats to HCH for 6 months markedly reduced ($P < 0.001$) the levels of phosphoinositides in erythrocyte membranes as well as in forebrain. It may be suggested from the present study that chronic HCH treatment might adversely affect vital membrane and cell functions modulated by phosphoinositides. Inositol phospholipids play a critical role in translating many hor-

monal and neurotransmitter signals at cell surface receptors into appropriate cellular responses. A marked decrease in the levels of phosphoinositides observed in chronically HCH-treated rat cerebrum could possibly derange the normal physiological responses brought about by phosphoinositide linked neurotransmitter receptors (Costa, 1990). It is of interest to note that lindane is known to cause behavioural changes (Rivera et al., 1990) and alterations in the levels of PI-linked neurotransmitters (Rivera et al., 1991). Although the steady state levels of phosphoinositides showed a trend to decrease in erythrocyte membranes obtained from rats treated with single doses of HCH for 24 h, the rate of synthesis of PI and polyphosphoinositides was increased, as judged from a marked increase in the incorporation of [2-^{3}H]inositol into PI and polyphosphoinositides. This indicates that the rate of hydrolysis of phosphoinositides could have been increased more than the increase in the synthesis resulting in a decrease in the level of phosphoinositides. Thus the phosphoinositide turnover and generation of second messengers from phosphoinositides were increased in the treated erythrocyte membranes.

The significant decrease in phosphoinositides observed in long-term HCH-treated rats could, possibly, be due to gradual accumulation of HCH or its decomposition product(s). It has been observed that HCH is present in rat erythrocytes (0.188 mg/ml erythrocytes) when the animals were fed 50 mg/kg HCH (commercial, 50% active component) 5 days a week for 30 days (Agrawal et al., 1992).

Even though the messenger systems in signal transduction work under suboptimal saturation levels so that small changes in inositol-phosphates may not be reflected quantitatively in genetic expression, the data on inositide levels in thymocytes, in the presence of mitogens and inhibitor, indicate feed back regulation (Taylor et al., 1984). Therefore, the present results may be indicative of signal transduction changes in HCH toxicity.

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References

Agrawal, D., Sultana, P. and Gupta, G.S.D. (1991) Oxidative damage and changes in the glutathione redox system in erythrocytes from rats treated with hexachlorocyclohexane. Food Chem. Toxicol. 29, 459–462.

Agrawal, D., Sultana, P. and Ray, P.K. (1989) Effect of pesticides on red bloodcells in vitro. Ind. Vet. Med. J. 13, 14–20.

Agrawal, D., Sultana, P., Gupta, G.S.D., Gopal, K., Khanna, R.N. and Anand, M. (1992) Effect of hexachlorocyclohexane on biochemical parameters of rats on a protein deficient diet. Toxicol. Environ. Chem. 35, 109–114.

Berridge, M.J. (1987) Inositol trisphosphate and diacylglycerol: Two interacting second messengers. Annu. Rev. Biochem. 56, 159–193.

Costa, L.G. (1990) The phosphoinositide/protein kinase C system as a potential target for neurotoxicity. Pharmacol. Res. 22, 393–408.

Fisher, D.B. and Mueller, G.C. (1971) Gamma-hexachlorocyclohexane inhibits the initiation of lymphocyte growth by phytohemagglutinin. Biochem. Pharmacol. 20, 2512–2518.

Fisher, S.K., Heacock, A.M. and Agranoff, B.W. (1992) Inositol lipids and signal transduction in the nervous system: An update. J. Neurochem. 58, 18–28.

Fukami, K., Furuhashi, K., Inagaki, M., Endo, T., Hatano, S. and Takenawa, T. (1992) Requirement of phosphatidylinositol 4,5-bisphosphate for α-actinin function. Nature 359, 150–152.

Gonzalez-Sastre, F. and Folch, J. (1968) Thin layer chromatography of phosphoinositides. J. Lipid Res. 9, 532–533.

Hayes, Jr., W.J. Pesticides Studied in Man. Williams and Wilkins, 428, Baltimore, USA, 1982, p. 211.

Hokin, L.E. (1985) Receptors and phosphoinositides generated second messengers. Annu. Rev. Biochem. 54, 205–234.

Hokin, M.R. and Brown, D.F. (1969) Inhibition by γ-hexachlorocyclohexane of acetylcholine stimulated phosphatidylinositol synthesis in cerebral cortex slices and cerebral cortex particulate fractions. J. Neurochem. 16, 475–483.

Michell, R.H. (1975) Inositol phospholipids and cell surface receptor function. Biochim. Biophys. Acta 415, 83–147.

Quist, E.E. (1982) Polyphosphoinositide synthesis in rabbit erythrocyte membranes. Arch. Biochem. Biophys. 219, 58–64.

Rivera, S., Sanfeliu, C. and Rodriguez-Farre, E. (1990) Behavioral changes induced in developing rats by an early postnatal exposure to lindane. Neurotoxicol. Teratol. 12, 591–596.

Rivera, S., Sanfeliu, C., Sunol, C. and Rodriguez-Farre, E. (1991) Regional effects on the cerebral concentration of noradrenaline, serotonin and dopamine in suckling rats after

a single dose of lindane. Toxicology 69, 43–54.

Steck, T.L. and Kant, J.A. (1974) Preparation of impermeable ghosts and inside-out vesicles from human erythrocyte membranes. Methods Enzymol. 31, 172–180.

Subramoniam, A. (1988) The phosphoinositides in transmembrane signalling. J. Sci. Ind. Res. 47, 587–601.

Subramoniam, A. and Subrahmanyam, D. (1982) Light induced changes in the phospholipid composition of Mycobacterium smegmatis ATCC 607. J. Gen. Microbiol. 128, 419–421.

Subramoniam, A., Goel, S.K., Pandya, K.P. and Seth, P.K. (1989) Influence of trichloroethylene treatment on phosphoinositides in rat brain. Toxicol. Lett. 49, 55–60.

Taylor, M.V., Metcalfe, J.C., Robin Hesketh, T., Smith, G.A. and Moore, J.P. (1984) Mitogen increase phosphorylation of phosphoinositides in thymocytes. Nature, 312, 462–465.